DESCRIPTION

GENES ENCODING PROTEINS REGULATING THE pH OF VACUOLES

Technical Field

The present invention relates to genes encoding proteins that regulate the pH of vacuoles, and the uses thereof.

Background Art

In the flower industry, the development of novel or varied cultivars of flowering plants is important, and flower color is one of the most important traits of flowers. Although cultivars of various colors have been bred using conventional breeding by crossing, it is rare that a single plant species has cultivars of all colors. Thus, there is a need for the development of cultivars having a variety of colors.

The main components of flower color are a group of flavonoid compounds termed anthocyanins. It is known that a variety of anthocyanins occur in plants, and the structure of many of them have already been determined. The color of anthocyanins depends partly on their structures. Progress has been made in the study on the enzymes and genes involved in the biosynthesis of anthocyanins, and in some studies molecular biological techniques and gene introductions into plants were used to change the structure of anthocyanins, leading to changes in the color of flowers (Holton and Cornish, Plant Cell, 7:1071 (1995); Tanaka et al., Plant Cell Physiol. 39:1119 (1998)). The color of anthocyanins also depends on the pH of the aqueous solution, and the same anthocyanin may appear blue when the pH of the aqueous solution is neutral to weakly alkaline (Saito and Honda, Genda Kadaku (Chemistry Today), May 1998, pp. 25).

It is also known that since anthocyanins are present in the vacuole of the cell, the pH of vacuoles has a

great impact on the color of flowers (Holton and Cornish, Plant Cell, 7 (1995); Mol et al., Trends Plant Sci. 3:212 (1998)). For example, in morning glory (Ipomea tricolor), it is known that the reason why red-purple buds bloom into blue flowers is that the pH of vacuoles in petal epithelium rises from 6.6 to 7.7 (Yoshida et al., Nature 373:291 (1995)).

It is thought that the vacuole of plant cells is regulated by vacuolar proton-transporting ATPase and vacuolar proton-transporting pyrophosphatase (Leigh et al., The Plant Vacuole (1997), Academic Press), but the mechanism of how these proton pumps are involved in the color of flowers has not been elucidated. It was also known that a sodium ion-proton antiporter (hereinafter referred to as Na^+-H^+ antiporter) exits in plant vacuoles and that the Na^+-H^+ antiporter transports sodium ions into vacuoles, depending on the proton concentration gradient between the outside and the inside of vacuoles, whereupon protons are transported outside of vacuoles resulting a reduced proton concentration gradient.

Furthermore, the Na^+-H^+ antiporter is thought to be a protein with a molecular weight of about 170,000. However, there are many unknown factors involved in the regulation of pH of vacuoles, and the mechanism of regulating the pH of vacuoles, in particular the petal vacuoles, is uncertain (Leigh et al., The Plant Vacuole (1997), Academic Press). The pH of plant vacuoles has never been artificially raised, nor have any industrially useful traits been obtained, and its association with flower color is unknown.

It is known that the Na^+-H^+ antiporter gene, with a molecular weight of about 70,000, has been cloned from Arabidopsis, and a yeast into which this gene was introduced has acquired salt tolerance (Gaxiola et al., Proc. Natl. Acad. Sci. USA 96:1480-1485 (1999)), but it is not known how this antiporter regulates the pH of vacuoles in plant cells or how it is associated with

flower color.

On the other hand, in petunias, seven loci are known to be involved in the pH regulation of petal vacuoles, and it has been proposed that the pH of petal vacuoles increases when one of them turns homozygously recessive (van Houwelingen et al., Plant J. 13:39 (1998); Mol et al., Trends Plant Sci. 3:212 (1998)). One of them, Ph6, has already been cloned and was found to be a kind of transcription regulating factor (Chuck et al., Plant Cell 5:371 (1993)), but the actual biochemical mechanism involved in the pH regulation of vacuoles is unknown.

In morning glory (Ipomea nil), the analysis of mutants revealed that a number of loci are associated with the color and shape of leaves and flowers and that 19 of them are highly mutable (Iida et al., Shokubutsu Saibo Kogaku Series (Plant Cell Engineering Series) 5 (1996) pp. 132, Shujunsha; Iida et al., Annal. New York Acad. Sci. (1999) pp. 870). Among them, the one locus defined by the recessive mutation that results in purple flowers instead of blue flowers is termed the Purple locus (T. Hagiwara, The genetics of flower colours in Phrarbitis nil. J. Coll. Agr. Imp. Univ. Tokyo 51:241-262 (1931); Y. Imai, Analysis of flower colour in Pharbitis nil. J. Genet. 24:203-224 (1931)), and one allele of mutable mutation that results in flowers that produce blue sectors in purple petals was termed purple-mutable (pr-m) (Imai, J. Coll. Agric. Imp. Univ. Tokyo 12:479 (1934)). The gene derived from the Purple locus is termed Purple gene.

The blue portion is believed to be derived from somatic reverse mutation from the recessive purple, and germ cell revertants can also be separated. An allele produced from the reverse mutation of these revertants are termed herein Purple-revertant (Pr-r). Such a classical method of genetic analysis had been performed on this Purple gene, but the identity of the Purple gene and its association etc. with the pH regulation of petal

vacuoles were totally unknown.

It is believed that if the pH of vacuoles could be modified, for example if the pH of vacuoles could be raised, flower color could be turned blue. Representative plant species that lack blue colors include roses, chrysanthemums, carnations, gerberas and the like, which are very important cut flowers. Though the importance of modifying pH of vacuoles has been recognized, the identities of proteins that regulate the pH of petal vacuoles are unknown and therefore the isolation of genes encoding them has been in great demand.

Disclosure of the Invention

The present invention provides a gene of a protein that regulates the pH of vacuoles in plant cells, preferably a gene of a protein that transports protons in vacuoles, more preferably a Na^+-H^+ antiporter gene. By introducing the gene of the present invention into a plant and allowing it to be expressed, flower color can be controlled and, preferably, can be turned blue.

Thus, the present invention provides a gene encoding a protein that regulates the pH of vacuoles. This gene is, preferably, a gene encoding a Na^+-H^+ antiporter, for example a gene encoding a protein that has the amino acid sequence as set forth in SEQ ID NO: 2, or a gene encoding a protein that has an amino acid sequence modified by the addition or deletion of one or a plurality of amino acids and/or substitution with other amino acids in the amino acid sequence as set forth in SEQ ID NO: 2 and that has an activity of regulating the pH of vacuoles; a gene encoding a protein that has an amino acid sequence having a identity of 20% or more with the amino acid sequence as set forth in SEQ ID NO: 2 and that has an activity of regulating the pH of vacuoles; or, a gene that hybridizes to part or all of a nucleic acid having a nucleotide sequence encoding the amino acid sequence as set forth in

SEQ ID NO: 2 under a stringent condition, and that encodes a protein having an activity of regulating the pH of vacuoles.

The present invention also provides a vector comprising the above gene.

The present invention also provides a host cell transformed with the above vector.

The present invention also provides a protein encoded by the above gene.

The present invention further provides a method of producing a protein that has an activity of regulating the pH of vacuoles, said method comprising culturing or growing the above host cell and then harvesting said protein from said host cell .

The present invention also provides a plant in which said gene or said vector has been introduced or an progeny thereof having the same property as said plant, or a tissue thereof.

The present invention also provides a cut flower of the above plant or an progeny thereof.

The present invention further provides a method of regulating the pH of vacuoles comprising introducing the above gene or the above vector into a plant or plant cells and then allowing it to be expressed.

The present invention further provides a method of controlling the flower color of plants comprising introducing the above gene or the above vector into a plant or plant cells and then allowing said gene to be expressed.

Brief Explanation of the Drawings

Fig. 1 is a drawing showing the structure of plasmid pSPB607.

Fig. 2 is a drawing showing the structure of plasmid pSPB608.

Fig. 3 is a drawing showing the structure of plasmid pINA145.

Fig. 4 is a drawing showing the structure of plasmid pINA147.

Best Mode for Carrying Out the Invention

The color of the petal of morning glory is blue when the locus Purple is dominant, and the blue petal turns purple when it is homozygously recessive. It is clear that the locus is associated with flower color but the mechanism thereof is unknown.

First, the chemical analysis of the pigments in the petal of the pr-m mutant and a revertant thereof detected no difference in the composition of the blue-colored morning glory from the reddish purple buds to the blue flowers accompanied by flowering is believed, as mentioned above, change in flower color of the blue flowers is not associated with to be caused by pH changes in the vacuole of petal cells. In the pr-m mutant, flowering is not associated with a color change to blue, and the pH of vacuoles of petal cells of flowers that bloomed was lower in the pr-m mutant than in pr-r. Thus, the Purple gene is considered to be a gene that regulates the pH of vacuoles of petal cells during flowering and thereby controls flower color. Accordingly, using a pr-m mutant, and a revertant thereof, by the transposon display method, fragments of genomic DNA containing the Purple gene sequence specifically present in pr-m were identified and then the Purple gene was identified. Surprisingly, the Purple gene thus obtained had a homology with the Na^+-H^+ antiporter from Arabidopsis etc., and, in the pr-m mutation, a transposon had been inserted in the 5'-untranslated region the Purple gene.

As the gene of the present invention, there can be mentioned, for example, one that encodes the amino acid sequence as set forth in SEQ ID NO: 2. It is known, however, that proteins having an amino acid sequence modified by the addition or deletion of one or a plurality of amino acids and/or substitution with other

amino acids also retain an activity equal to that of the original protein. Thus in accordance with the present invention, a protein that has an amino acid sequence modified by the addition or deletion of one or a plurality of amino acids and/or substitution with other amino acids in the amino acid sequence as set forth in SEQ ID NO: 2, and a gene encoding said protein, are encompassed in the present invention as long as the protein is a protein that has an activity of regulating the pH of vacuoles.

The present invention also relates to a gene that hybridizes to the nucleotide sequence as set forth in SEQ ID NO: 1, a nucleotide sequence encoding the amino acid sequence as set forth in SEQ ID NO: 2, or a nucleotide sequence encoding part of these nucleotide sequences at a stringent condition, for example at 5 × SSC and 50°C, and that encodes a protein having an activity of regulating the pH of vacuoles. As used herein, a suitable hybridization temperature varies with the nucleotide sequence and the length of the nucleotide sequence, and when, for example, a DNA fragment comprising 18 bases encoding 6 amino acids is used as a probe, a temperature of 50°C or lower is preferred.

Genes selected, based on such hybridization, include those obtained from nature, for example from plants such as petunia and torenia, but a gene derived from sources other than plants may be used. Genes selected based on hybridization may be cDNA or genomic DNA.

The Na^+-H^+ antiporter genes form a superfamily (Debrov et al., FEBS Lett. 424:1 (1998)), and have an amino acid homology of 20% or more (Orlowski et al., J. Biol. Chem. 272:22373 (1997)).

Thus, the present invention relates to a gene encoding a protein that has an amino acid sequence with a homology of about 20% or more, preferably 50% or more, for example 60% or 70% or more, and that has an activity of regulating the pH of vacuoles.

A gene having an intact nucleotide sequence is obtained, as specifically illustrated in Examples, by, for example, screening cDNA libraries. DNA encoding a protein having a modified amino acid sequence can be synthesized by commonly used site-directed mutagenesis or the PCR method based on DNA having an intact nucleotide sequence. For example, a DNA fragment that is to be modified may be obtained by restriction enzyme treatment of the intact cDNA or genomic DNA, which is used as a template in the site-directed mutagenesis, or by the PCR method using primers in which desired mutation has been introduced to obtain a DNA fragment in which the desired modification has been introduced. Thereafter, the mutated DNA fragment may be ligated to a DNA fragment encoding another portion of the enzyme of interest.

Alternatively, in order to obtain DNA encoding a protein comprising a shortened amino acid sequence, an amino acid sequence longer than the amino acid sequence of interest, for example, DNA encoding the full-length amino acid sequence, may be cleaved with a desired restriction enzyme, and when the resultant DNA fragment was found not to encode the entire amino acid sequence of interest, a DNA fragment comprising the sequence of the lacking portion may be synthesized and ligated thereto.

The present invention is not limited to a gene encoding a protein that has an activity of regulating the pH of vacuoles derived from morning glory, but the sources may be plants, animals, or microorganisms, and all they need is to have a topology that pumps protons out of the vacuole.

By expressing the obtained gene using a gene expression system in Escherichia coli or yeast and determining the activity, it can be confirmed that the gene obtained encodes a protein that has an activity of regulating the pH of vacuoles. Furthermore, by expressing said gene, a protein, the gene product, having an activity of regulating the pH of vacuoles can be

obtained. Alternatively, a protein can also be obtained that has an activity of regulating the pH of vacuoles using an antibody against the amino acid sequence as set forth in SEQ ID NO: 2, and a protein that has an activity of regulating the pH of vacuoles derived from other organisms can be cloned using an antibody.

Thus, the present invention also relates to a recombinant vector comprising the above-mentioned gene, specifically an expression vector, and a host cell transformed with said vector. As a host, there can be used a prokaryotic or eukaryotic organism. As a prokaryotic organism, for example, there can be used such a common host as a bacterium belonging to the genus Escherichia such as Escherichia coli, a bacterium belonging to the genus Bacillus such as Bacillus subtilis, and the like. As a eukaryotic host, there can be used a lower eukaryotic organism, for example an eukaryotic microorganism such as a fungus, a yeast or a mold.

As yeast, there can be mentioned a microorganism belonging to the genus Saccharomyces such as Saccharomyces cerevisiae, and as a mold, there can be mentioned a microorganism belonging to the genus Aspergillus such as Aspergillus oryzae and Aspergillus niger, and a microorganism belonging to the genus Penicillium. Furthermore, animal cells or plant cells can be used: as animal cells, there can be used cell lines derived from mouse, hamster, monkey, human and the like. Insect cells such as silkworm cells or adult silkworms per se can also be used as hosts.

The vectors of the present invention may contain expression regulatory regions such as a promoter, a terminator, an origin of replication, and the like, depending on the type of the host into which said vector is to be introduced. As promoters for bacterial expression vectors, there can be used commonly used promoters such as trc promoter, tac promoter, lac

promoter, and the like; as promoters for yeasts, there can be used the glyceraldehyde-3-phosphate dehydrogenase promoter, PHO5 promoter, and the like; and as mold promoters, there can be used amylase promoter, trpC promoter, and the like.

As promoters for animal cell hosts, there can be used viral promoters such as SV40 early promoter, SV40 late promoter, and the like. The construction of expression vectors may be performed according to conventional methods using restriction enzymes, ligase, etc. The transformation of host cells can also be performed according to conventional methods.

Host cells transformed with the above expression vectors may be cultured, cultivated or bred, and from the culture the desired protein can be recovered and purified according to conventional methods such as filtration, centrifugation, cell disruption, gel filtration chromatography, ion exchange chromatography, and the like.

The present invention also relates to a plant or its progenies or tissues thereof of which hue of color has been controlled by introducing a gene encoding a protein that has an activity of regulating the pH of the vacuoles, specifically a Na^+-H^+ antiporter gene. They may be cut flowers in shape. Using a gene encoding a protein that has an activity of regulating the pH of vacuoles obtained by the present invention, the pumping of proton into the cytoplasm from the vacuole and the pumping of sodium ion into the vacuole can be performed, so that anthocyanins accumulated in the vacuole can be turned blue and, as a result, the flower color can be turned blue.

It is also possible to lower the pH of vacuoles by suppressing the expression of the gene of the present invention. With the state-of-the-art technology, it is possible to introduce a gene into plants, and allow the gene to be expressed in a constitutive or tissue-specific

manner, and also to suppress the expression of the gene of interest by the antisense method or the co-suppression method.

Examples of plants that can be transformed include, but not limited to, roses, chrysanthemums, carnations, snapdragons, cyclamens, orchids, lisianthus, freesias, gerberas, gladioluses, gypsophilas, kalanchoes, lilies, pelargoniumas, geraniums, petunias, torenias, tulips, rice, barley, whieat, rapeseeds, potatoes, tomatoes, poplars, bananas, eucalyptuses, sweet potatoes, soy beans, alfalfas, lupins, corns, and the like.

Examples

The present invention will now be explained in further details with reference to the following Examples. Molecular biological techniques used were performed according to Molecular Cloning (Sambrook et al., 1989), unless otherwise specified.

Example 1. Obtaining a germ cell revertant

Obtaining a germ cell revertant has already been reported (Iida et al., Shokubutsu Saibo Kogaku Series (Plant Cell Engineering Series) 5 (1996) pp. 132, Shujunsha; Iida et al., Annal. New York Acad. Sci. (1999) pp. 870; Inagaki et al., Plant Cell, 6:375 (1994); Inagaki et al., Theor. Appl. Genet. 92:499 (1996)).

Morning glory having the genotype (Pr-r/pr-m) (Iida et al., pp. 870; Inagaki et al., Plant Cell, 6:375 (1994); Inagaki et al., Theor. Appl. Genet. 92:499 (1996)) was subjected to self-fertilization and the seeds of the progeny were planted. The flowers of the self-fertilized progeny were observed to select individuals that bloom with blue flowers by back mutation. Furthermore, in this self-fertilized progeny of the germ cell revertant, it was proved whether it is homozygous or heterozygous based on whether or not isolates that bloom with purple flowers can be obtained. Those having the genotype (Pr-r/Pr-r) and (pr-m/pr-m) were selected.

Example 2. Anthocyanins in the petals of revertants

Anthocyanins contained in morning glory are mainly heavenly blue anthocyanin and several other anthocyanins (Lu et al., Phytochemistry 31:659 (1992)). When the open petals of the Pr-r/Pr-r strain and the pr-m/pr-m strain obtained in Example 1 were similarly analyzed, the anthocyanins contained in both of them were almost identical.

A cellophane tape was stuck to the front side of a petal and then peeled off to recover one layer of epithelium, from which the cell liquid was scraped with a scalpel etc., which was then centrifuged to obtain juice. The pH of the juice was measured using the Horiba B212 pH meter (Horiba Seisakusho). pH of the petal epithelium of the Pr-r/Pr-r strain was about 7.1 whereas that of the pr-m/pr-m strain was about 6.5. This result indicates that the change in flower color by mutation of purple was not due to the structure of anthocyanins but to the change of vacuolar pH.

Example 3. Isolation of a genome fragment specifically present in pr-m

For the isolation of a gene, the transposon display method (Frey et al., Plant J. 13:717 (1998); Van den Broeck et al., Plant J. 13:121 (1998)) or a similar method (Dosho et al., Shokubutsu Saibo Kogaku Series (Plant Cell Engineering Series) 7 (1997) pp. 144, Shujunsha) was used to search for DNA bands that were present in the pr-m/pr-m strain and the Pr-w/pr-m strain but not in the Pr-r/Pr-r strain or in the wild strain. Since Tpn1-related transposon is thought to be mainly associated with mutability in morning glory, special note was given to the Tpn1-related transposon.

Specifically, chromosomal DNA was extracted from the pr-m/pr-m strain, and 125 ng of it was digested with MseI in 20 μl. To the digested DNA was added 80 pmole of MseI adaptor (obtained by annealing 5'-GACGATGAGTCCTGAG-3' (SEQ ID NO: 3) and 5'-TACTCAGGACTCAT-3' (SEQ ID NO: 4))

in 25 μl at 20°C for 2 hours. After keeping it at 75°C for 10 minutes, it was stored at -20°C. After diluting this ten-fold, 2 μl was used as a template, which was PCR-amplified using 4.8 pmole of TIR primer (5'-TGTGCATTTTTCTTGTAGTG-3' (SEQ ID NO: 5), this includes the inverted terminal repeat of the transposon Tpn1) and 4.8 pmole of MseI primer (5'-GATGAGTCCTGAGTAA-3') (SEQ ID NO: 6) in 20 μl.

PCR was performed with Taq polymerase (Takara Shuzo) for 20 cycles with one cycle comprising 94°C for 0.5 minute, 56°C for 1 minute, and 72°C for 1 minute, and the volume was diluted ten-fold. Two μl of it was used as a template in a PCR using 4.8 pmole of TIR+N primer (5'-TGTGCATTTTTCTTGTAGN-3' (SEQ ID NO: 7) N=A, C, G or T. Four different species were synthesized instead of a mixture) and 4.8 pmole of MseI+N primer (5'-GATGAGTCCTGAGTAAN-3' (SEQ ID NO: 8) N=A, C, G or T. Four different species were synthesized instead of a mixture. The 5'-end was labeled with fluorescein (using Amersham Pharmacia Biotek, Vistra fluorescence 5'-oligo labeling kit)) in 20 μl.

Reactions were performed for combinations of primers to a total of 16 reactions. PCR was performed for 13 cycles with one cycle comprising 94°C for 0.5 minute, 65°C (with a decrement of 0.7°C for each cycle) for 1 minute, and 72°C for 1 minute, and further for 13 cycles with one cycle comprising 94°C for 0.5 minute, 56°C for 1 minute, and 72°C for 1 minute. A similar procedure was performed for chromosomal DNA obtained from the Pr-r/Pr-r strain, subjected to electrophoresis using a sequence gel of the DNA Sequencer 377 (PE Biosystems Japan), and the bands were detected using FMBIOII (Takara Shuzo).

When bands derived from the Pr-r/Pr-r strain and the pr-m/pr-m strain were compared, an about 130 bp DNA fragment was specifically expressed in the strain having

pr-m. The 130 bp DNA fragment was recovered, and amplified by PCR (for 30 cycles with one cycle comprising 94°C for 0.5 minute, 56°C for 1 minute, and 72°C for 1 minute) using 20 pmole TIR primer and 20 pmole MseI primer, which was then subcloned into the pGEM-T vector (Promega Corporation), and then the nucleotide sequence was determined. The sequence was

5'-TGAGCATTTTTCTTGTAGTG CTGAGATTTTCCTCCATTTGTCTGAAGCTCTTCATCCTTCAACAC TACCCCCACATCTCACCTTTCAAG GTCCAATCTTTATCATTCATCT TTACTCAGGACTCATCGTC-3'

(SEQ ID NO: 9) (the single-underlined portion corresponds to a used primer, the double-underlined portion corresponds to an exon, and the rest corresponds to an intron). After the sequence as set forth in SEQ ID NO: 9 was used as a probe in Northern analysis, a transcription product of about 2.3 kb was found in the bud of morning glory having Pr-r, but a corresponding transcription product was not found in the pr-m/pr-m strain. Thus, it can be seen that this 2.3 kb transcription product corresponds to the Purple gene.

Example 4. Isolation of cDNA

About 6 million clones of a cDNA library (Inagaki et al., Plant Cell 6:375 (1994)) derived from the wild strain morning glory (Pr-w/Pr-w) were screened using the 130 bp DNA fragment as a probe, with a result that two positive clones were obtained. One of these clones had a 2237 bp cDNA, among which a 1626 bp-long open reading frame was observed (SEQ ID NO: 1). The predicted amino acid sequence had an identity of 29.3% and 73.4% with the Na^+-H^+ antiporter of yeast and Arabidopsis, respectively (Nhx1 and AtNhx1, respectively, Gaxiola et al., Proc. Natl. Acad. Sci. USA 96:1480-1485 (1999)).

The result revealed that the Purple gene of morning glory encodes a Na^+-H^+ antiporter. Incidentally, although the Na^+-H^+ antiporter obtained from Arabidopsis is attracting attention as a protein that gives salt resistance to yeast, this is the first time that an association of the Na^+-H^+ antiporter with flower color

was observed.

Example 5. Complementation experiment of yeast Na^+-H^+ antiporter

The predicted amino acid sequence encoded by the Purple gene of morning glory has a homology with those of the Na^+-H^+ antiporters of yeast and Arabidopsis. Thus, in order to confirm whether the Purple gene product of morning glory can function as a Na^+-H^+ antiporter protein, a complementation experiment was performed using a yeast Na^+-H^+ antiporter mutant.

First, the following two DNA fragments were synthesized:

CBSC1-Linker (22 mer) 5'-CGA TAG ATC TGG GGG TCG ACA T-3' (SEQ ID NO: 12)

CSBD2-Linker (22 mer) 5'-CGA TGT CGA CCC CCA GAT CTA T-3' (SEQ ID NO: 13)

From these two fragments, a linker having restriction enzyme sites ClaI-BglII-SalI-ClaI is formed. A plasmid pINA145 (Fig. 3) was constructed by inserting the above linker according to a standard method into the ClaI site of the pYES2 vector (Invitrogen Corporation) so that the BglII site is located at the URA3 gene side. A plasmid pINA147 (Fig. 4) was constructed by ligating a 2 kb DNA fragment obtained by digesting plasmid pJJ250 (Jones and Prakash, Yeast 6:363-366 (1990)) with BamHI and SalI to plasmid pINA145 digested with BglII and SalI. Plasmid pIAN151 was constructed by ligating Purple cDNA thereto under the control of the GAL 1 promoter of plasmid pINA147. pINA147 and pIAN151 were transformed respectively to the yeast R101 strain which is a mutant strain of the Na^+-H^+ antiporter. Due to the mutation of the Na^+-H^+ antiporter, the yeast R101 strain cannot grow on a 400 mM NaCl-added APG medium (Nass et al., J. Biol. Chem. 272:26145 (1997); Gaxiola et al., 96:1480-1485 (1999)). The pINA147-transformed R101 strain could not grow either, and only the pIAN151-transformed R101 strain could grow on the 400 mM NaCl-added APG medium. The

result has shown that the gene product of the morning glory Purple gene has the Na^+-H^+ antiporter function.

Example 6. Construction of an expression vector in plants

With 10 ng of morning glory Purple cDNA as template, PCR was performed using synthetic primers PR-5 (5'-GGGATCCAACAAAAATGGCTGTCGGG-3') (SEQ ID NO: 10) and PR-3 (5'-GGGTCGACTAAGCATCAAAACATAGAGCC-3') (SEQ ID NO: 11). The polymerase used was Taq polymerase (Toyoboseki), and the reaction was performed, after reaction at 95°C for 45 seconds, for 25 cycles with one cycle comprising 95°C for 45 seconds, 50°C for 45 seconds, and 72°C for 45 seconds, and then further reacted at 72°C for 10 minutes. An about 1.6 kb DNA fragment obtained was ligated to pCR2.1-Topo (Clontech) to make pCR-purple. It was confirmed that there were no errors due to PCR in the nucleotide sequence of Purple cDNA on this plasmid.

pBE2113-GUS (Mitsuhara et al., Plant Cell Physiol. 37:49 (1996)) was digested with SacI and blunt-ended. Then a XhoI linker (Toyoboseki) was inserted thereto, and the plasmid obtained was termed pBE2113-GUSx. This was digested with EcoRI and HindIII to obtain an about 2.7 kb DNA fragment, which was ligated to the HindIII and EcoRI digest of pBinPLUS, and the plasmid obtained was termed pBEXP.

On the other hand, an about 1.2 kb DNA fragment obtained by digesting pCGP484 (Kohyo (National Publication of Translated Version) No. 8-511683) with HindIII and XbaI, an about 1.6 kb DNA fragment obtained by digesting pCR-purple with XbaI and SalI, and an about 13 kb DNA fragment obtained by digesting pBEXP with HindIII and XhoI were ligated to obtain pSPB607 (Fig. 1). This plasmid is a binary vector for use in the Agrobacterium-mediated transformation of plants, and on this plasmid Purple cDNA is under the control of a chalcone synthase promoter derived from snapdragon and a nopaline synthase terminator derived from Agrobacterium.

An about 0.8 kb DNA fragment obtained by digesting pCGP669 (Kohyo (National Publication of Translated Version) No. 8-511683) with HindIII and BamHI, an about 1.6 kb DNA fragment obtained by digesting pCR-purple with BamHI and SalI, and an about 13 kb DNA fragment obtained by digesting pBEXP with HindIII and XhoI were ligated to obtain pSPB608 (Fig. 2). This plasmid is a binary vector for use in the Agrobacterium-mediated transformation of plants, and on this plasmid Purple cDNA is under the control of a chalcone synthase promoter derived from petunia and a nopaline synthase terminator derived from Agrobacterium.

By transforming plants using the expression vectors thus obtained, the pH of vacuoles can be regulated and thereby flower color can be controlled.

Example 7. Isolation of a homologs of the Purple gene

cDNA libraries derived from the petals of petunia (Petunia hybrida cv. Old Glory Blue), Nierembergia (Nierembergia hybrida cv. NB17), and Torenia (Torenia hybrida cv. Summerwave Blue) were each constructed using the cDNA synthesis kit (Stratagene, USA). The method of construction was as recommended by the manufacturer. About 200,000 clones each were screened according to a standard method. For washing the membrane, an aqueous solution of 5 × SSC and 0.1% SDS was used and the incubation was performed three times at 50°C for 10 minutes. Among the positive clones obtained, the nucleotide sequence of the longest clone was determined for each clone. The nucleotide sequence of the clone of Petunia and the corresponding amino acid sequence are shown in SEQ ID NO: 14 and 15, the nucleotide sequence of the clone of Nierembergia and the corresponding amino acid sequence are shown in SEQ ID NO: 16 and 17, and the nucleotide sequence of the clone of Torenia and the corresponding amino acid sequence are shown in SEQ ID NO: 18 and 19. Homologs of the Purple gene of Petunia, Nierembergia, and Torenia had an identity on the amino

acid level of 75%, 76%, and 71%, respectively, with the morning glory Purple gene.

Since the amino acid sequence of the Na^+-H^+ antiporter encoded by the morning glory Purple gene and that of the Na^+-H^+ antiporter encoded by Arabidopsis AtNhx 1 are about 73% identical, the homologs of the Purple gene of Petunia, Nierembergia, and Torenia obtained are judged to encode the Na^+-H^+ antiporter.

Example 8. Isolation of the clone of morning glory Purple chromosome

After chromosomal DNAs of a mutant morning glory (pr-m/pr-m) and a revertant morning glory (Pr-r/Pr-r) were cleaved with BglII, they were electrophoresed on a 0.8% agarose gel, and were subjected to genomic Southern analysis with cDNA of morning glory Purple as a probe. As a result, an about 7.5 kb band that was not present in the mutant morning glory was detected in the revertant morning glory.

After 50 μg of chromosomal DNA of the wild type morning glory (Pr-w/Pr-w, the KKZSK2 strain) was digested with BglII, it was electrophoresed on a 0.8% agarose gel. An about 7-9 kb fragmently was recovered, from which DNA was extracted using the GENECLEAN III KIT (B10101). This DNA was ligated to the λ Zap express vector (Stratagene, USA), which was screened with cDNA of morning glory Purple as a probe. The determination of nucleotide sequences of positive clones obtained revealed that, on this about 7.5 kb DNA fragment, there was a region from about 6.3 kb upstream of the Purple promoter to midway in exon 3. For this sequence, a sequence up to the initiation codon of the Purple gene is shown in SEQ ID NO: 20.

It has been demonstrated that the expression of the Purple gene is strongly induced only at about 24 hours before the flowering of morning glory, and that the expression of the Purple gene is suppressed by insertion

of a transposon into the 5'-untranslated region. From this, it is clear that the promoter region of the Purple gene obtained contains a factor needed for the expression of the Purple gene in a developmental stage-specific and organ-specific manner in the petals of morning glory. By placing the gene of interest downstream of this promoter region, the expression of the gene of interest can be regulated in a developmental stage-specific and organ-specific manner.

Industrial Applicability

The gene obtained in the present invention was found, for the first time, to be involved in controlling the pH of vacuoles and flower color. By expressing the gene of the present invention on the flower petals, the pH of vacuoles can be increased and thereby the flower color can be turned blue. Furthermore, by suppressing the expression of the gene of the present invention, the pH of vacuoles can be lowered and thereby flower color can be turned red. As the gene encoding a protein that regulates the pH of vacuoles, there can be used not only those derived from morning glory obtained in the present invention but also similar genes derived from other organisms.